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Electron transfer in pheophytin *a*-modified reaction centers from *Rhodobacter sphaeroides* (R-26)

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The major part (> 90%) of bacteriopheophytin *a* in reaction centers (RCs) of *Rhodobacter sphaeroides* was substituted by plant pheophytin *a*. In modified RCs the photochemical formation of $P^+Q_a^-$ occurs with with a quantum efficiency of 79%. The intermediary state P^+I^- displayed a recombination time constant of 1.5 ns, and the electron transfer from I^- to Q_a was characterized by a time constant of 540 ps. On the basis of spectral properties of P^+I^- for native and modified RCs, it was suggested that bacteriopheophytin, as well as bacteriochlorophyll monomers located in L protein branch, have a transition at 545 nm with approx. equal extinction coefficients. Accordingly, the state P^+I^- in modified RCs is proposed to consist of a thermodynamic mixture of P^+BL^- (~ 80%) and P^+Phe^- (~ 20%).

Reaction center; Primary electron donor and acceptor; Electron transfer; Bacteriopheophytin; Pheophytin; *Rhodobacter sphaeroides*

1. INTRODUCTION

A key question related to the mechanism of primary light conversion in bacterial reaction centers (RC) is connected to the formation of the primary charge transfer state with participation of the primary electron donor, P, monomeric bacteriochlorophyll (BChl) B and bacteriopheophytin (BPhe) H. It was found by means of ps and fs spectral analysis [1–3], as well as by detailed analysis of fs kinetics at different wavelengths [3,4], that the primary charge transfer state P^+BL^- is formed within 3 ps (here and below, indexes L and M indicate the protein subunits L and M, however, the indexes A and B can be used as well, respectively), which is converted in the sub-ps time domain into P^+HL^-. Some kinetic data with lower signal-to-noise ratio were interpreted to indicate direct formation of P^+HL^- from P* without intermediary formation of P^+Bl^- [5]. Hole-burning spectroscopy of RCs has indicated the possibility that the first step of the charge transfer is the fastest one (~ 200 fs) [6].

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Abbreviations: BChl, BPhe, Chl and Phe, bacteriochlorophyll *a*, bacteriopheophytin *a*, chlorophyll *a* and pheophytin *a*, respectively; BL and BM, bacteriochlorophyll monomers located in L and M protein subunits, respectively; HL and HM, bacteriopheophytins in L and M subunits, respectively; P, primary electron donor, bacteriochlorophyll dimer; Q_a and Q_b, primary and secondary quinons, respectively; Q_y and Q_x, first and second electronic transitions in Chl-like molecules, respectively; RCs, reaction centers.

Detailed analysis at $T > 100$ K of the relaxed state P^+I^- (where I is an electron acceptor complex) with blocked electron transfer to the primary quinone (Q_a) [7] and at $T > 220$ K of the relaxed state Cyt^+PI^- [7,8], have shown that I^- is not simply HL^- but includes in addition some perturbation of monomeric BChls reflected by a blue shift and by the bleaching of part of the Qy transition of BL. Illumination of RCs at low temperature leads to the appearance of a specific HL^- difference spectrum with 'simple' blue shift of BL transition in P^+I^- spectrum [7] and in the accumulated spectrum of Cyt^+PI^- [7–9]. These data were interpreted in terms of thermal equilibration and relaxation at $T > 220$ K of the states P^+BL^- and P^+HL^- (or Cyt^+PBL^- and Cyt^+PHL^-) on one hand [7] and of RCs conformational changes [8]. The former interpretation meets the problem of temperature independence of the transition at 545 nm. In this case it has to be suggested that both HL and BL have a transition in this spectral region.

A new approach to the study of this aspect has been introduced [10,11], based on the modification of pigments in the RCs. Particularly, the replacement of BPhe by pheophytin *a*(Phe) could create a barrier for electron transfer through the Phe mediator and reveal the charge transfer state, P^+BL^-, more clearly. In this work we used the replacement of BPhe by plant Phe in *Rhodobacter sphaeroides* RCs as described by Scheer et al. [11] and found that the formed P^+I^- is mostly represented by the state, P^+BL^-, with a recombination time constant of 1.5 ns, whereas there is only relatively small contribution (~ 20%) from P^+Phe^-. The data suggest that HL as well as BL have the transition at 545 nm with approx. equal extinction coefficients.

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2. MATERIALS AND METHODS

RCs from *R. sphaeroides* (R-26) were isolated by treatment of chromatophores with lauryldimethylamine oxide (LDAO) followed by purification with DEAE-cellulose chromatography [12]. Phe was extracted from nettle and purified as described elsewhere (see [13]). The purity of Phe preparations was checked by absorption spectroscopy.

Pigment exchange experiments were performed as described by Scheer et al. [11]. Briefly, RCs solubilized in 10 mM Tris-HCl (pH 8.0), 0.1% LDAO, 10% methanol, containing a 20-fold excess of Phe were incubated in the dark at 42°C for 90 min. Then the excess of free pigment was removed by chromatography on DEAE-cellulose. As a control, RCs from the stock solution solubilized in the same medium without Phe were submitted to analogous incubation and purification procedures.

Absorption spectra were measured with a Hitachi UV-160 spectrophotometer. Light-induced absorbance changes in the ps, μs and ms time domains were measured as described earlier [1,12]. For ps difference absorbance spectra measurements a OMA-2 optical multichannel analyzer (EG&G PARC, Princeton) was used. The intensity of excitation pulses at 605 nm was close to 25% of saturating level to avoid overexcitation of the pigments and was the same for all measurements. The stirring of the sample was used to excite the different part of the solution during measurements.

3. RESULTS

Fig. 1A compares the absorption spectrum of native RCs from *R. sphaeroides* (R-26) (curve 1) with that of RCs which are modified by Phe (curve 2) RCs. In agreement with results obtained earlier [11] the main amount (> 90%) of BPhe is replaced by Phe in modified RCs, as indicated by the decrease of BPhe bands at 760 nm (~ 10 times) and 535 nm (~ 10 times) and the formation of new absorption bands of Phe at 672 nm and 505 nm. The band at 545 nm is slightly modified. This band is two times (1.6–2.6 depending on preparation) larger with respect to the 505-nm band in contrast to Phe solution [13] where the latter is slightly larger. Modified RCs display photochemical activity. Under illumination the state P^+Q^- forms with a quantum efficiency of 79% with added exogenous quinone (72% without additions). The spectrum of P^+Q^- (Fig. 1B) is characterized by the same features as with native RCs except for the decrease of the red shift of the BPhe band at 760 nm by factor of > 5 and the appearance of the red shift of the Phe band at 672 nm. The kinetics of P^+Q^- recombination (τ ~ 80 ms) are similar to those of native RCs (not shown).

Figs. 2A and 3 show the difference absorption spectra for a 30 ps delay with respect to the center of the excitation pulse at 605 nm for native (dashed line) and Phe-modified (solid line) RCs in the near infra-red (Fig. 2A), red (Fig. 3A) and green (Fig. 3B) regions in the presence of preoxidized quinones. The modified RCs' spectra are characterized by bleaching of the 870 nm, 800 nm, 670 nm and 540 nm bands, which is qualitatively as expected for the formation of P^+I^-. Comparison of P^+I^- spectra obtained for modified and native RCs shows the following features: (i) the same amplitude of the bleaching at 870 nm; (ii) a considerable decrease (~ 5 times) in modified RCs of the bleaching of HL band at 760 nm characteristic for native RCs (Fig. 2A); (iii) at least two times more bleaching at 800 nm with modified RCs; (iv) the same amplitude of bleachings near 542 nm with both preparations; (v) the appearance of a new bleaching at 670 nm in modified RCs.

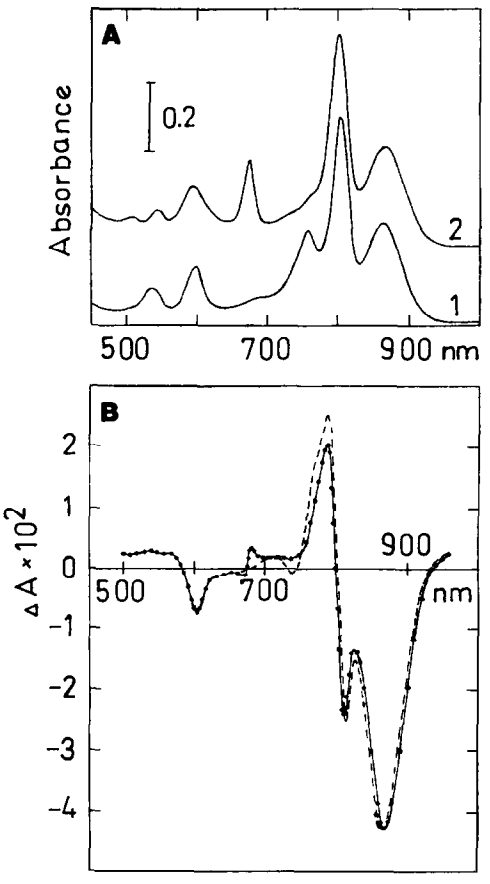

Fig. 1. (A) Room-temperature absorption spectra of RCs from *R. sphaeroides* (R-26). Native RCs (curve 1); RCs after exchange of BPhe molecules by Phe (see section 2) (curve 2) with the same absorption at 870 nm as native ones. RCs were suspended in 10 mM Tris-HCl (pH 8.0), 0.1% LDAO, 250 mM NaCl. The spectrum of control RCs (see section 2) was similar to that of native RCs (curve 1). (B) Difference (light-minus-dark) absorption spectra for photooxidation of P measured at room temperature with phosphoroscope set-up for native (dashed line) and Phe-modified (solid line) RCs.

Fig. 2A (inset) shows the kinetics of ΔA at 870 nm in modified RCs. There is partial (~ 25%) relaxation of the bleaching within less than 1 ns, and then the relaxed state is maintained for at least 50 μs.

The kinetics of the bleaching at 542 nm reflecting the formation and relaxation of I^- in modified RCs in monoexponential with a time constant of 540 ± 50 ps (Fig. 3C).

Fig. 2B shows that in the presence of prereduced quinones in modified RCs the ΔA spectrum at 30 ps delay (solid) is similar to that with preoxidized quinones, however, the decay of ΔA at 870 nm, as well as

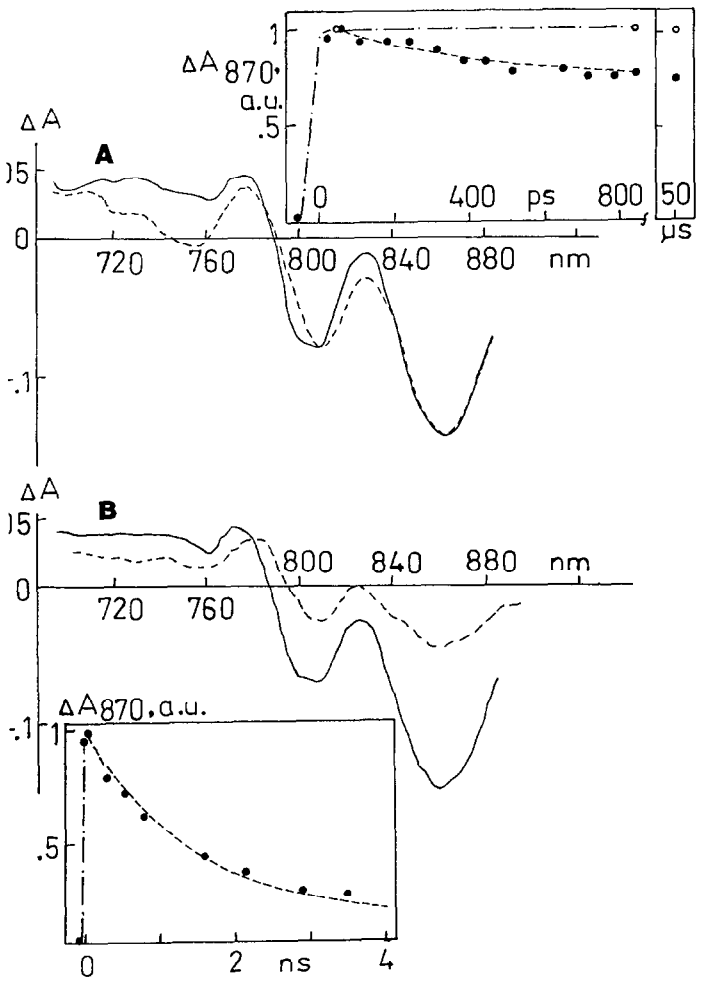

Fig. 2. (A) Difference absorption spectra of native (dashed line) and Phe-modified (solid line) RCs from *R. sphaeroides* (R-26) in the presence of UQ6 (2 molecules per RC) and 0.5 mM sodium ascorbate, measured at 30 ps after the center of a non-saturating excitation pulse at 605 nm. Inset shows the kinetics of ΔA at 870 nm for native (○) and modified (●) RCs. The points at 50 μs were measured using the μs set up. The dashed line shows the calculated kinetics with parameters indicated in the text. (B) The spectra of ΔA of modified RCs in the presence of 50 mM sodium ascorbate, 200 mM DAD and continous illumination to prereduce the quinones in RCs, measured at 30 ps (solid line) and 3 ns (dashed line) after excitation at 605 nm. Inset shows the kinetics of ΔA at 870 nm. The dashed line shows the simulated exponential decay kinetics with time constant of 1.5 ns for 90% of ΔA. 10% of ΔA does not decay within 4 ns. (20°C, A at 870 = 0.5 for all measurements).

at other wavelengths, is faster and displays a time constant of 1.5 ± .3 ns for 90% of ΔA (10% of ΔA does not decay within 4 ns).

4. DISCUSSION

The results presented show that in Phe-modified RCs of *R. sphaeroides* (R-26) the photoinduced charge separation leading to the formation of P^+Q^- is observed. The spectral features of P^+Q^- in modified RCs reveal that the Phe molecules are affected in terms of electrochromic shift by charges of P^+ and/or Q^-, and therefore are in the vicinity of the cofactors of the RCs. The ps formation of the state P^+I^- is also observed in modified RCs. This state is converted into the state P^+Q^- within 540 ps with quantum efficiency of 79% in the presence of added quinones. A lower quantum efficiency with respect to native RCs is probably due to the faster recombination of the state P^+I^- (time constant of 1.5 ns) as compared to native RCs (~ 13 ns [7]). The dashed curve displayed in the inset of Fig. 2A represents the calculated kinetics of ΔA at 870 nm assuming two pathways for the decay of P^+I^-: (i) formation of $P^+Q_a^-$ with a time constant of 540 ps; (ii) relaxation to the ground state with a time constant of 1.5 ns. These time constants were obtained by independent measurements of ps kinetics of ΔA at 542 nm with preoxidized (540 ps) and at 870 nm with prereduced quinones (1.5 ns) (Figs. 2B and 3B).

The nature of I^- in modified RCs is the subject of further discussion. The considerable decrease of bleaching at 760 nm in the state P^+I^- in modified RCs is consistent with the replacement of BPhe by Phe. Then two possibilities are obvious: (i) the I^- spectrum reflects formation Phe^-, (ii) the I^- spectrum reflects formation

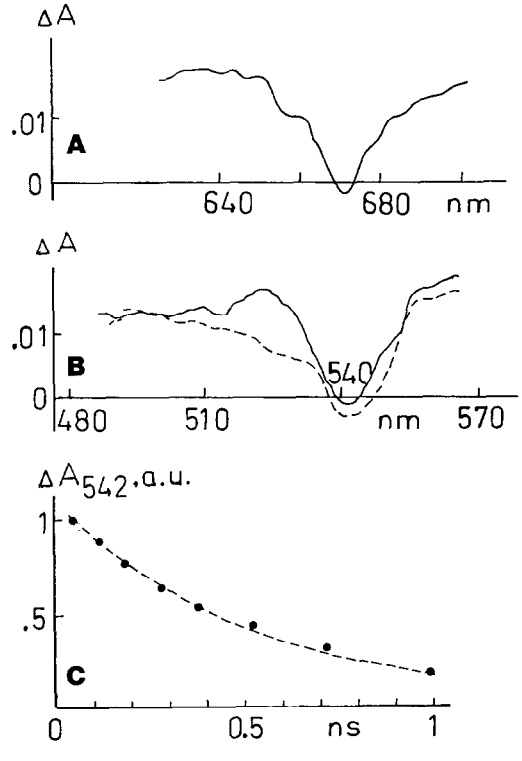

Fig. 3. (A,B) The spectra of ΔA of native (dashed line in B, native not shown in A) and Phe-modified (solid line) RCs from *R. sphaeroides* (R-26) in the presence of 0.5 mM sodium ascorbate, measured at 30 ps after a non-saturating excitation pulse at 605 nm. (C) Kinetics of ΔA at 542 nm in modified RCs. The dashed line shows simulated exponential kinetics with a time constant of 540 ps (20°C, A at 870 = 0.5 for all measurements).

of BL^-. Both possibilities meet problems in interpretation.

According to model spectra for Phe^- formation, the extinction coefficient for absorbance changes in the red region is ~ 5 times larger with respect to that in the green region [13]. The same features were observed for the accumulation of Phe^- in photosystem II RCs [14]. In both cases bleaching of the 535–545 nm band is accompanied by bleaching of the vibronic band at 505–515 nm with almost equal amplitudes. The situation in modified R-26 RCs is quite different. In the P^+I^- spectrum the band at 670 nm displays a differential extinction coefficient equal to or smaller than that of the band at 542 nm (Fig. 3). No absorbance changes in the region of 505–515 nm are observed, although the absorbance spectrum of Phe in modified RCs (Fig. 1) is consistent with that of model systems. To explain these discrepancies we need to suggest that the spectral properties of Phe^- formed upon charge separation in modified RCs are different from those in model systems and photosystem II RCs and also from those found for the absorption spectrum of Phe in modified RCs. The reason for this is not clear.

On the other hand, the BChl and Chl spectra in model systems display a Q_x transition at variable wavelengths. The Q_x transition is observed near 570–580 nm for a 5-coordinate Mg atom which is displaced out of the plane of the nitrogen atoms (N1–4) by ~ 0.35 Å [15–17]. For a 6-coordinate Mg atom located in the N1–4 plane the Q_x transition is shifted to 610–640 nm [15,16]. This shows the possibility for a change in the Q_x transition frequency depending on the position of Mg in Chl-like molecules. The decrease of symmetry of the molecule is accompanied by blue shift of the Q_x transition. In fact, the coordinates of Mg in BL show [18] that the position of Mg is also displaced from the center of BChl by 0.22 Å which exceeds the shift observed for other BChl molecules in RCs [18] and in model systems [17] by ~ 0.1 Å. Therefore one can assume that BL might have the Q_x transition at an even shorter wavelength, namely near 545 nm, as observed in ps spectra (Figs. 2 and 3).

If this assumption is valid, the consequences for an understanding of the primary charge separation in RCs may be considered. First of all, the bleaching near 545 nm is not an indicator of HL^- alone. The P^+I^- spectrum observed in modified RCs should be mainly (~ 80%) a P^+BL^- spectrum because the bleaching at 670 nm is 4–5 times smaller than expected from the 870- and 542-nm bleachings under the same conditions. This is consistent with additional bleaching near 800 nm in the P^+I^- spectrum of modified RCs (Fig. 2). The time constant for the recombination of the primary state P^+BL^- (~ 7 ns) was suggested to be shorter than that for the secondary state P^+HL^- (~ 21 ns) [7] to explain the temperature dependence of recombination rate of mixing state P^+I^- in native RCs. In fact, the time constant for recombination of P^+BL^- is approx. 1.5 ns in the absence of BPhe according to Fig. 2B (it can be different in the presence of BPhe due to additional relaxation of the medium after formation of P^+HL^-).

The small bleaching of the 670-nm band of Phe in modified RCs might reflect some fraction (~ 20%) of P^+Phe^- populated in modified RCs which support electron transfer to Q_a. The population of only a small fraction of P^+Phe^- is probably due to the higher energy of this state compared to that of P^+BL^- and is consistent with a decrease of the rate constant for forward electron transfer in modified RCs (1/540 ps^{-1}) with respect to native RCs (1/200 ps^{-1}).

Following the above assumptions the ratios for differential extinction coefficients can be roughly estimated from difference absorption spectra of P^+I^- for modified and native RCs. The ratios are: $\varepsilon P870/\varepsilon BL800 = 1.22$; $\varepsilon BL800/\varepsilon BL542 = 4.58$; $\varepsilon HL760/\varepsilon HL542 = 2.43$; $\varepsilon BL800/\varepsilon HL760 = 2.46$; $\varepsilon BL542/\varepsilon HL542 = 1.3$. One can see that both BL and HL have approx. the same extinction coefficients for the 542 nm transition. Other ratios (± 30%) are in reasonable correlation with the model absorbance spectra of pigments.

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